# Influence of SIRT6 Knock Down on Stability of β-Catenin in Non- Small Cell Lung Cancer Cell Line

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**Abstract**
*Sirtuins are a class of NAD+-dependent proteins that play a key role in regulating lifespan and preserving metabolic equilibrium. According to reports, sirtuin 6 (SIRT6) may have an oncogenic effect on lung cancer. Nevertheless, it is unclear exactly how SIRT6 functions at the molecular level in human lung cancer. For lung cancer to continue, Wnt signalling must be properly regulated. It is still mostly unclear how Wnt signalling is epigenetically regulated in NSCLC. As a result, we looked at how crucial the SIRT6 function is in A549 and H460. In human NSCLC, SIRT6 was substantially expressed. NSCLC cell viability was markedly reduced when SIRT6 was silenced. β-catenin is less phosphorylated when SIRT6 is knocked down. These results suggest that SIRT6 inhibition may increase the quantities of active β-catenin protein in NSCLC, which in turn may influence WNT signaling.*
**Keywords: SIRT6, Lung Cancer, β-Catenin**

## Introduction

Cancer develops from the transformation of typical cells into tumour cells in a multistage process that progresses from a pre-cancerous lesion to a malignant tumour (Cao, p. D et al., 2011). These changes or mutations are usually genetic and may be aided by tobacco smoke, hormones, certain viruses, and environmental hazards such as radiation, ultraviolet radiation, and carcinogenic chemicals.

Epigenetics is the study of genetic control by factors other than a person's DNA sequence. Epigenetic changes or expressed, while others can activate or deactivate genes and determine which proteins are transcribed. Changes worldwide in the epigenetic landscape are characteristic of cancer. It is unknown if epigenetic anomalies, in addition to genetic mutations, play a role in cancer, a disease long believed to be hereditary in nature.

Recent advances on the rapidly evolving field of cancer epigenetics have revealed extensive reprogramming of every epigenetic component (Julia M. Wagner et al., 2010).

Heritability of gene expression patterns is mediated by epigenetic modifications such as cytosine base methylation in DNA, posttranslational modifications of histone proteins, and nucleosome positioning along the DNA. The complement of these modifications, known as the epigenome, provides a mechanism for cellular diversity

by regulating genetic information that cellular machinery can access. According to Sharma et al. (2010), improper maintenance of heritable epigenetic marks can lead to the improper activation or inhibition of different signaling pathways, which can cause disease states like cancer.

For many years, cancer cells have been shown to have epigenetic modifications. Only lately has it been acknowledged that aberrant DNA methylation is not just a result of the transformation process, but rather plays a causative role in carcinogenesis, despite being defined as an early step in the process. This insight led to the discovery that cancer cells also exhibit other epigenetic modifications, including modifications to the posttranslational state of histones. The core DNA sequence is not permanently altered by epigenetic modifications, which are transitory in nature. Tumor suppressor genes and important oncogenes exhibit these epigenetic modifications. and transcription factors, resulting in the initiation and progression of cancer.

A potent genetic technique for determining the genes implicated in particular biological processes in human cells and model organisms is RNA interference (RNA) gene silencing. It is now feasible to quickly discover novel therapeutic targets and look into their roles in carcinogenesis because to the development of large-scale gene knockdown utilizing siRNA libraries (Guo, W et al., 2013). In mammalian cell-based systems, RNA interference (RNAi) is currently routinely utilized to investigate known signal transduction pathways and find new genes. Many studies using siRNA libraries have identified Akt-cooperative kinases (Morgan-Lappe, S. et al., 2006), genes influencing TRAIL-induced cell death (Aza-Blanc, P. et al., 2003), and NF-B signalling, a novel familial cylindromatosis tumour suppressor gene (Brummelkamp, T. R. et al., 2003). Other than these modest research projects

In mammalian cells, RNA interference (RNAi) has quickly spread to larger-scale gene knockdown research (Colas, A.R et al., 2012, Lee, S. K et al., 2012). Retroviral-based siRNA libraries that target approximately one-third of the human genome (Berns, K. et al., 2004), human proteasome function (Paddison et al., 2004), and novel tumour suppressor pathways have been used to identify genes implicated in p53-mediated cell cycle arrest.

Malignant tumors are characterized by their invasive development and propensity to spread. One of the most important phases in the invasive development process is the tumor cells' separation from their intercellular connections. Numerous cell adhesion molecules (CAMs) are crucial to this procedure. The immunoglobulin superfamily, cadherins, integrins, and selectins are some of the most researched CAMs. To attach to another cell, the calcium-dependent homotypic glycoproteins that make up cadherins create dimeric zipper-like adhesive structures at the cell membrane. The actin cytoskeleton and E (epithelial)-cadherin are connected by cytoplasmic proteins called á-, â-, and ã-catenin.

E-cadherin's cytoplasmic domain interacts with â-catenin instead of á-catenin directly, as á-catenin mediates the contact with the actin filament network. Empirical data indicates that disruption of the E-cadherin/catenin complex plays a pivotal role in the process of differentiation loss and invasion initiation.In a small percentage of human malignancies, mutations in the E-cadherin gene, which is located on chromosome 16q22.1, have been discovered. According to our unpublished observations, we were unable to find this gene's mutations in any non-small-cell lung malignancies (NSCLC). It has been demonstrated that the E-cadherin/catenin complex is controlled by various factors at several, primarily non-genomic, levels.

## Materials and Methods

### Maintenance of Cells

### Cell Lines

- A549: Cell line for lung cancer
- Large cell lung cancer cell line NCI-H460

### Cell Culture

The National Centre for Cell Sciences (NCCS), located in Pune, India, is where the lung cancer cell lines A549 and NCI-H460 were acquired. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), which was enhanced with 10 % Fetal Bovine Serum (FBS) and 1 % antibiotics (penicillin, streptomycin, and amphotericin B). Every cell was grown in a $CO_2$ incubator with 5 % $CO_2$ and a humidified environment at 37°C. To sustain the cells in an exponential phase, stock cultures were subcultured until all of the cells reached 80 % confluency. The cells were then collected using trypsin-EDTA and seeded in a tissue culture flask after being rinsed with phosphate-buffered saline (PBS).

### Cell Culture Reagents

### Incomplete Medium

Himedia Laboratories, Mumbai, India provided the DMEM medium containing HEPES (4-(2-hydroxyethy1)-1-piperazine ethane sulfonic acid) buffer, glucose, glutamine, sodium bicarbonate, and sodium pyruvate.

### Serum

10 % Fetal bovine serum (FBS) was acquired from Mumbai, India's Himedia Laboratories.

### Antibiotics

We bought amphotericin B, streptomycin, and penicillin from Himedia Laboratories in Mumbai, India.

### Complete Medium (DMEM with 10 % FBS)

To 90 ml of DMEM and 1 ml of antibiotics, 10 ml of FBS were added to create 100 ml of growth medium/complete media. It was kept in an inoffensive container.

### Chemicals

Hi-Media Laboratories, located in Mumbai, India, provided the trypsin, EDTA, disodium hydrogen phosphate, sodium chloride, potassium chloride, and potassium dihydrogen phosphates. The analytical grade of all other chemicals utilized in this experiment were obtained from SRL and Hi-Media Laboratories in Mumbai, India, and they were all related to cell culture.

### 0.1 % Trypsin-EDTA

In 100 ml of 1xPBS, 0.1 g of trypsin and 0.1 g of EDTA were dissolved.

### 1X Phosphate Buffered Saline (PBS) pH 7.4

500 milliliters of sterile double-distilled water were used to dissolve 0.74 g of disodium hydrogen phosphate ($Na_2HPO_4$), 4 g of sodium chloride (NaCl), 0.1 g of potassium chloride (KCl), and 0.12 g of potassium dihydrogen phosphate ($KH_2PO_4$). After using 0.1 N NaOH to get the pH down to 7.4, the mixture was sterile filtered (0.22 µm), and it was refrigerated.

**Plastic-Wares**

Purchases from Tarson's Products, Kolkata, India, included tissue culture flasks, tissue culture plates, centrifuge tubes, serological pipettes, tips, and other items.

**Passaging the Cells**

After the cells reached confluence, they were separated using the trypsin-EDTA solution in the following manner: The culture's Medium was aspired to. After swiftly aspirating the flask once more, 2 cc of 1X PBS was used to rinse it. After adding 1 ml of trypsin-EDTA solution, the mixture was incubated at 37°C for approximately 3–5 minutes, or until the cells began to separate from the surface. As soon as the cells were free, the trypsinated medium containing the cells was centrifuged for three minutes at 1000 rpm using a serological pipette. With caution, the medium was aspirated. The pipette tip was carefully removed from the tube where the cells were pelleted.

Through retro pipetting, the cells were gradually suspended in 10 % FBS-containing new DMEM liquid. A drop of the cell suspension was applied to the Neuberger hemocytometer's coverslip's edge. By capillary action, the drop was allowed to run under the coverslip. Air bubbles were prevented from entering the liquid by being careful not to "force" it. Next, using a microscope, the cells from the E1, E2, E3, E4 and E5 squares were counted. After that, the cells were carefully resuspended in new growth medium and put into sterile T-25 flasks. The medium's volume was then increased to 5 ml using the growth medium/flask combination.

**Transfection Efficiency of siRNA Oligonucleotides and Optimization of Transfection**

NSCLC cell lines were seeded in 1X106 per well of 6-well plates without antibiotics one day prior to transfection after achieving 60 % confluence. SIRT6 siRNA6 oligos and negative control siRNA at a concentration of 25 nM were employed on the transfection day. Diluted siRNA (Tube A) and Lipofectamine 3000 (transfection reagent) (Tube B) were produced in serum-free medium and placed in different tubes. Tube A: 150 µl of serum-reduced OPTI-MEM medium diluted with 2 µl of 25nM siRNA oligonucleotides. Tube B: 150 µl of OPTI-MEM serum-reduced medium with 6 µl of lipofectamine 3000. Each tube was incubated for five minutes. Next, the diluted siRNA oligonucleotide and diluted Lipofectamine 3000 were combined, gently mixed, and incubated for twenty-five minutes at room temperature.

Following incubation, cells and antibiotic-free media were introduced dropwise to six-well plates containing siRNA and Lipofectamine 3000 complexes. Cells containing the transfection complex were incubated at 37°C with 5 % oxygen, and they were kept alive for 48 hours following transfection.

**Transfection of Sirt6 SiRNA and Negative Control**

After achieving 60 % confluency, lung cancer cells were planted into a six-well plate (1X106). SIRT6 siRNA (sense: 5'GAAUGUGCCAAGUGUAAGAtt3', antisense: 5'UCUUACACU UGGCACAUUCtt-3') and its negative control siRNA (AM4611) were acquired from Invitrogen in the United States for use in siRNA transfection. Following the manufacturer's instructions, the cells were transfected using Lipofectamine 3000 reagent (Invitrogen, USA) at a concentration of 25nM. The cells were kept alive for 48 hours following transfection, with the lipofectamine reagent alone acting as a sham control. Western blotting and RT-PCR were used to assess the effectiveness of the transfection.

## Reverse Transcriptase PCR Analysis

### Total RNA Isolation

Takara RNAiso plus reagent (Takara Bio Inc., Japan) was used to isolate total RNA. In summary, siRNA-transfected A549 and NCI-H460 cells are used to silence unsilenced A549 and NCI-H460 cells after the cells have been twice washed with ice-cold PBS. The cells were lysed directly in a culture plate using 1 milliliter of TRIzol reagent, collected into 2 milliliter centrifuge tubes, and mixed with 0.2 milliliter of chloroform. The cells were vortexed rapidly for 5 minutes and then incubated for 20 minutes at room temperature. Following incubation, the samples were centrifuged for 15 minutes at 4°C at 12,000 rpm, and the upper aqueous phase—which contains total RNA—was carefully removed and placed in a fresh 1.5 ml centrifuge tube.

The aqueous phase was separated from the total RNA by employing isopropyl alcohol. After gently mixing the aqueous phase with 0.5 ml of isopropyl alcohol by inverting the tubes, the mixture was incubated for 10 minutes at room temperature. Following incubation, the samples were centrifuged at 10,000 rpm for 10 minutes at 4°C, and the supernatant was carefully removed. The RNA pellet was then washed by adding 1 ml of 75 % ethanol, gently mixed, and the tubes were inverted once more. After centrifugation, all leftover ethanol was removed, and the tubes were inverted again, allowing the RNA pellet to air dry for 10 minutes. 50 µl of nuclease-free water was added to dissolve the RNA pellet, which was then kept in a freezer at -20°C for further use in tests.

### RNA Quantification and cDNA Construction

Using the Eppendorf bio spectrophotometer, total RNA was measured. Only cDNA synthesis was conducted on the A260/A280 ratio <1.80 and the A260/A230 ratio <0.5 samples. For the creation of cDNA, 2µg of RNA was utilized. Using the Prime Script TM RT reagent kit (Catalog number: RR037A-Takara Bio Inc, Japan), cDNA was created. To prepare a 0.2 µl PCR tube for 20 µl of cDNA, add 5X Prime Script buffer (-4.0 µl), Prime Script RT Enzyme (-1µl), 50 µM Oligo dT primer (1.0 µl), Random 6 mars (-1µl), RNA (-2 µg), and Nuclease-free water up to 13 µl. The RNA-Primer mixture was incubated in the following circumstances: Takara PCR thermal cycler dice (Takara): 37°C for 15 minutes, 85°C for 5 seconds, and final hold at 4°C Synthesized cDNA was stored at-20°C for further experiments.

### RT-PCR

Using the Emerald Amp RT PCR master mix (Catalog number: RR310A-Takara Bio Inc, Japan) and the Takara PCR thermal cycler dice, the mRNA expression levels were measured by RT-PCR (Takara Bio Inc, Japan). To perform a 10 µl RT-PCR reaction, add 5 µl of 2X PCR master mix, 1 µl of cDNA, 1 µl of Forward Primer, 1 µl of Reverse Primer, and 2 µl of nuclease-free water to PCR tubes. Gently mix the reagents and incubate for 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds.

Gel documentation was done using Gelstan 1012 (Mediccare Scientific, Chennai, India) and 2% agarose gel electrophoresis was used to evaluate the expression of each gene of interest, normalized to a housekeeping gene (β-ACTIN). Using ImageJ software (National Institutes of Health, Bethesda, MD, USA), the band intensities were measured.

## Western Blotting

### Total Protein Isolation

A549 and NCI-H460 cells that had been transfected with siRNA to silence them as well as their negative controls were lysed using 1 milliliter of RIPA (Radio Immunoprecipitation assay) lysis buffer (pH 7.4+0.1) that contained 10 microliters of PMSF (200 mM), 10 microliters of

sodium orthovanadate (100 mM), and 10 microliters of protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Using a cell scraper, the cells were removed from the plates and placed into microcentrifuge tubes. The tubes were then placed in the ice for fifteen minutes and vortexed for five minutes. Following incubation, the cells were centrifuged for 20 minutes at 4°C at 12,000 rpm.

After being collected in a fresh tube, the cleared supernatant was centrifuged once more for ten minutes at 4°C at 12,000 rpm. For later usage, the cleaned supernatant was gathered and aliquoted. Samples of isolated proteins were kept in a freezer set at -20°C until use in additional tests.

**Protein Estimation**

The concentration of total protein was determined by applying Lowry's technique (Lowry et al., 1951). At 660 nm, the absorbance of the standards and samples was measured using BSA (bovine serum albumin) as the standard.

**Separation of Protein by SDS-Gel Electrophoresis and Detection**

After being resuspended in 6X protein loading buffer, the quantified protein (50 µg per lane) was separated using 12% SDS-polyacrylamide gel electrophoresis (100 V, 2.30h). It was then transferred onto a nitrocellulose membrane (150 V for 1.30h) using Towbin buffer (25 mM Tri's base, 190 mM glycine, 20 % methanol, pH: 8.3) from Bio-Rad, Hercules, CA, USA. To prevent nonspecific antibody binding, the membranes were treated for one hour at room temperature with blocking buffer, which was made freshly and consisted of 5 % skimmed milk powder in TBS-T (20 mM Tris, 136 mM NaCl, 0.1 Tween 20, pH 7.6). Primary monoclonal antibodies against mice and rabbits were diluted 1:10,000 and incubated for an entire night at 4°C.

Membranes were washed (3 times for 5 min) with TBS-T and TBS. And then further the membranes were then treated three times for five minutes with TBS-T and TBS after being incubated with goat anti-rabbit or anti-mouse secondary antibody (cell Signalling Technology, Danvers, MA, USA). The 200 µl BCIP/NBT solution (Merck Millipore, Bedford, MD, USA) was used to identify bands. Bio-Rad Gel doc XR plus (Bio-Rad, Hercules, CA, USA) was used to observe the bands, and ImageJ software (NIH) was used to quantify the band intensities.

**Results & Discussion**

**Silencing of SIRT6 Stabilizes the β-CATENIN by Decreasing the Phosphorylation**

In line with the earlier findings, we speculate that the suppression of WNT in NSCLC cell lines is caused by increased β-CATENIN expression that is controlled by SIRT6. According to our findings, SIRT6 knockdown lowers the β-phosphorylation status of the β-CATENIN protein, which may lead to an increase in the protein's stability in NSCLC cell lines. Using an immunoblotting test, we evaluated if SIRT6 suppression might raise the DNMT1 protein level. The results showed that SIRT6 knockdown increased the amount of active β-CATENIN.

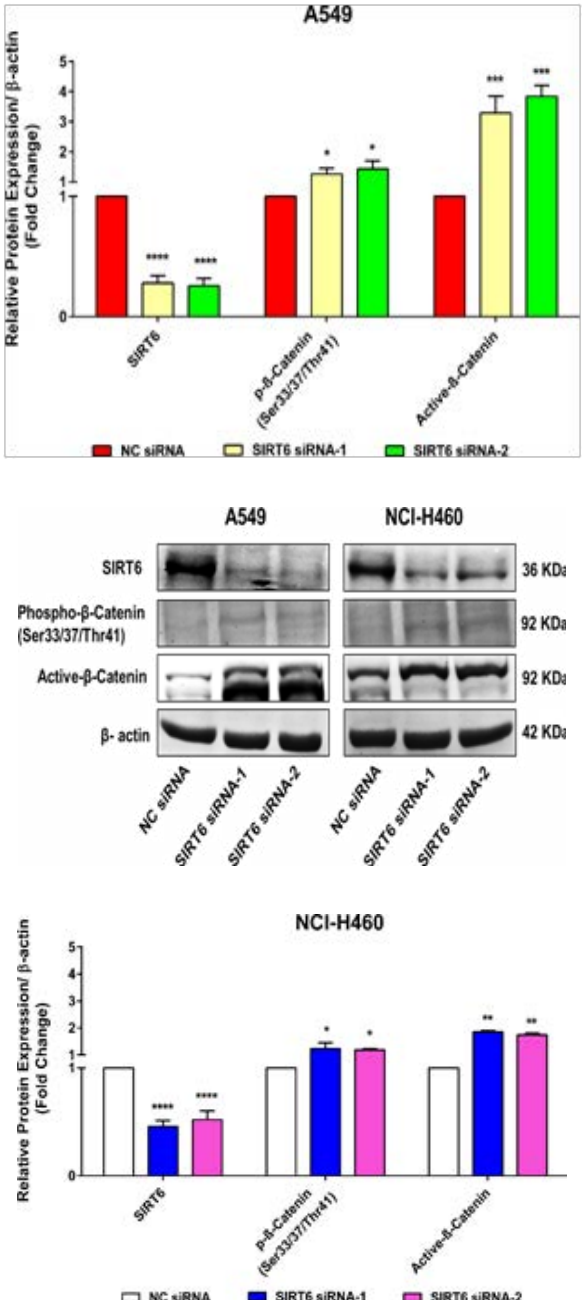


**Figure 5.1 Status of β Catenin upon SIRT6 Knockdown (SIRT6 siRNA 1 and SIRT6 siRNA 2 in NSCLC Cell Line**

Wnt/βcatenin signaling is evolutionarily conserved and required for embryonic development and tissue homeostasis. Wnt/βcatenin signaling is frequently reported to participate in the development and progressions of various types of tumors. This The signaling system is crucial for intercellular communication and has been remarkably conserved throughout evolution. A growing body of research suggests that improving the expression of Wnt/β-catenin signaling elements, including

as receptors and downstream targets, is critical for reversing the EMT phenotype and overcoming drug resistance. In this investigation, si-SIRT6 was used to transduce NSCLC cells. We discovered a substantial rise in β-Catenin protein levels, indicating that SIRT6 inversely controlled this pathway in NSCLC cells. Therefore, our results showed that SIRT6 silencing increases β-catenin/ Wnt signaling by posttranslational modification.

## Conclusion

A family of NAD+-dependent proteins called sirtuins is important in maintaining metabolic balance and controlling lifespan. It has been reported that sirtuin 6 (SIRT6) has an oncogenic influence in lung cancer. Nevertheless, it is unclear exactly how SIRT6 functions at the molecular level in human lung cancer. For lung cancer to continue, Wnt signaling must be well regulated. It is still mostly unclear how Wnt signaling is epigenetically regulated in NSCLC. As a result, we looked at how crucial the SIRT6 function is in A549 and H460. In human NSCLC, SIRT6 was substantially expressed. NSCLC cell viability was markedly reduced when SIRT6 was silenced. β-catenin is less phosphorylated when SIRT6 is knocked down. These findings imply that SIRT6 suppression may control WNT signaling by raising the amounts of active β-catenin protein in NSCLC.

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